*J. Mol. Biol.* (1977) **113**, 237–251

# Labeling Deoxyribonucleic Acid to High Specific Activity *in Vitro* by Nick Translation with DNA Polymerase I

PETER W. J. RIGBY†, MARIANNE DIECKMANN, CARL RHODES AND PAUL BERG

*Department of Biochemistry*
*Stanford University Medical Center*
*Stanford, Calif. 94305, U.S.A.*

*(Received 13 December 1976)*

Circular (e.g. simian virus 40) and linear (e.g. λ phage) DNAs have been labeled to high specific radioactivities ($>10^8$ cts/min per μg) *in vitro* using deoxynucleoside [α-$^{32}$P]triphosphates (100 to 250 Ci/mmol) as substrates and the nick translation activity of *Escherichia coli* DNA polymerase I. The reaction product yields single-stranded fragments about 400 nucleotides long following denaturation. Because restriction fragments derived from different regions of the nick-translated DNA have nearly the same specific radioactivity (cts/min per $10^3$ bases), we infer that nicks are introduced, and nick translation is initiated, with equal probability within all internal regions of the DNA. Such labeled DNAs (and restriction endonuclease fragments derived from them) are useful probes for detecting rare homologous sequences by *in situ* hybridization and reassociation kinetic analysis.

## 1. Introduction

Nucleic acid hybridization is the most powerful method for detecting and quantitating specific RNA and DNA sequences. Reassociation of complementary strands to form duplex structures can be carried out in a variety of ways: with either RNA or DNA in solution and single-stranded DNA immobilized in agar (Bolton & McCarthy, 1962), on nitrocellulose filters (Gillespie & Spiegelman, 1965) or sheets (Southern, 1975), or chemically linked to an insoluble matrix (Noyes & Stark, 1975); alternatively, annealing can occur in solution and the resulting double-stranded nucleic acid can be adsorbed to hydroxylapatite (Bernardi, 1965), recovered by isopycnic centrifugation (Hall & Spiegelman, 1961), or separated from single strands by digestion with specific endonucleases (Sutton, 1971). Whatever the annealing procedure and whatever the means used to quantitate the hybridization, one of the partners in the hybridization reaction is isotopically labeled.

By monitoring the reassociation kinetics of a radioactively labeled, randomly sheared DNA probe in the presence and absence of DNA or RNA samples, the quantity of specific nucleotide sequences in those samples can be readily determined (Gelb *et al.*, 1971; Reed *et al.*, 1976). The sensitivity of this type of measurement is often a function of the specific radioactivity of the DNA probe. For example, Gelb *et al.* (1971), who were the first to employ the procedure to detect and quantitate the

† Present address: Department of Biochemistry, Imperial College of Science and Technology, London, SW7 2AZ, England.

number of integrated simian virus 40 (SV40) DNA copies per SV40-transformed cell, required about 5 mg of cell DNA to detect one to five copies per cell (5 to 25 ng SV40 DNA) with a probe of specific activity $10^6$ cts/min per $\mu$g. Only 25 $\mu$g of cell DNA would suffice if the labeled SV40 DNA probe contained $2 \times 10^8$ cts/min per $\mu$g.

During the past several years we have developed and refined a procedure for labeling DNA to high specific radioactivity *in vitro*. This method has since been adopted in a number of laboratories besides our own for hybridization experiments requiring more sensitivity than can be achieved with nucleic acids labeled *in vivo* (Schachat & Hogness, 1973; Wensink *et al.*, 1974; Glover *et al.*, 1975; Jaenisch & Mintz, 1974; Maniatis *et al.*, 1975; Botchan *et al.*, 1976; Green *et al.*, 1976*a*,*b*; Reed *et al.*, 1976; Frenkel *et al.*, 1976; Smith *et al.*, 1976). The method utilizes the ability of *Escherichia coli* DNA polymerase I to catalyze a "nick translation" reaction (Kelly *et al.*, 1970), i.e. to couple the sequential addition of nucleotide residues to the 3′-hydroxyl terminus of a nick with the elimination of nucleotide units from the nick's 5′-phosphoryl terminus. With labeled deoxynucleoside triphosphates as substrates, the pre-existing, unlabeled nucleotides in the DNA template are replaced by radioactive ones; the specific activity of the DNA depends upon the specific activity of the substrates and the extent of nucleotide replacement.

In this paper we describe the preparation of the high specific activity deoxynucleoside [$^{32}$P]triphosphate substrates, the nick translation reaction with SV40 and $\lambda$ phage DNAs and some characteristics of the labeled products. Labeled, nick-translated SV40 DNA can be used to detect and quantitate DNA sequences present at the level of one SV40 DNA copy per haploid mouse genome using microgram quantities of cell DNA.

## 2. Materials and Methods

### (a) *Viral and cellular DNAs*

Closed circular SV40 DNA was isolated from infected CV-1 cells by the procedure previously described (Shenk *et al.*, 1976). Bacteriophage $\lambda$cI857Sam7 DNA was isolated according to Thomas & Davis (1974) except that phage protein was removed by extraction with phenol. *Drosophila melanogaster* DNA (Schachat & Hogness, 1973) was provided by D. J. Finnegan and SV40-transformed mouse cell DNA was isolated by a modification of the procedure of Gross-Bellard *et al.* (1973).

### (b) *Enzymes*

*Bam*HI, *Hin*dIII and *Hin*cII restriction endonucleases were prepared by Biogel A 0·5M chromatography, ammonium sulfate fractionation, phosphocellulose and DEAE-cellulose chromatography as described by R. J. Roberts (personal communication); *Hin*fI restriction endonuclease by the same procedure omitting the ammonium sulfate fractionation; and *Hae*III restriction endonuclease by the same procedure omitting the DEAE-cellulose chromatography (Roberts *et al.*, 1975). *Hpa*I restriction endonuclease was prepared by the method of Sharp *et al* (1973), *Eco*RI restriction endonuclease by a modification (M.-T. Hsu, T. A. Landers & P. Berg, unpublished results) of the method of Greene *et al.* (1974) and $S_1$ nuclease by the method of Vogt (1973).

*E. coli* DNA polymerase I, obtained from A. Kornberg, was the homogeneous preparation of Jovin *et al.* (1969). We have also used other preparations of DNA polymerase I, purified by the procedure of Jovin *et al.* (1969); these were equally efficient in catalyzing the nick translation reaction and gave similar reaction products. The enzyme is stored at a protein concentration of 3 mg/ml in 0·1 M-potassium phosphate (pH 7) in liquid nitrogen. Portions (10 $\mu$l) of this solution are thawed, diluted with 10 $\mu$l glycerol and stored at $-20$°C; under these conditions the enzyme has a useful life of about 6 months. Enzyme which has been stored for too long at $-20$°C incorporates label effectively but extensive

strand displacement occurs and the DNA product contains variable (10 to 40%) amounts of rapidly reannealing DNA. This may well be due to the 5′ to 3′ exonuclease activity of the enzyme being more labile than the polymerase activity.

We have no experience with commercial preparations of DNA polymerase I. However, others have successfully used our procedures with commercial enzyme (Botchan *et al.*, 1976).

DNAase I (electrophoretically purified) was purchased from Worthington Biochemical Corporation. Stock solutions (1 mg/ml in 0·01 N-HCl) were stored at −20°C in 50-μl portions. The DNAase was activated by thawing a portion into 0·45 ml of 10 mM-Tris·HCl (pH 7·5), 5 mM-$MgCl_2$, 1 mg bovine serum albumin/ml and incubating this solution at 0°C for 2 h. Immediately before use the DNAase was diluted with the same buffer to give a DNAase concentration of 133 ng/ml. Since there is some variation in the activity of commercial DNAase preparations, stocks of DNAase solution were prepared and the amount needed to introduce the desired number of nicks was determined experimentally for each batch. The enzyme appears to be stable when stored in this way and so the predetermined amount of DNAase can be used in subsequent experiments.

### (c) *Synthesis of deoxynucleoside [α-$^{32}P$]triphosphates*

Several commercial vendors, for example New England Nuclear, supply deoxynucleoside [α-$^{32}P$]- and [$^3H$]triphosphates that are suitable for the nick translation reaction. But to obtain the highest specific activity DNA preparations, and also for economic reasons, we generally prepare the labeled substrates by a modification of a procedure first described by Symons (1969).

Approximately 50 mCi of carrier-free $^{32}P$-labeled $H_3PO_4$ (in 0·05 M-HCl) and sufficient $H_3PO_4$ to give a spec. act. of 200 to 250 Ci/mmol are mixed, applied to a column (0·5 ml) of Dowex-50 ($H^+$ form) and eluted with 2 ml of water. After drying by rotary evaporation and resuspension of the residue in sufficient triethylamine solution to neutralize the HCl, the sample is evaporated twice more and resuspended in water. After the removal of a sample for determination of the specific activity the solution is dried down again and an excess (50 μmol of each) of deoxyadenosine and deoxycytosine hydrochloride, suspended in acetonitrile plus sufficient triethylamine to neutralize the hydrochloride, is added. The mixture is evaporated to dryness twice, with intermittent suspension in acetonitrile, and, after introduction of dry air, 0·3 ml of dimethylsulfoxide together with 21 μmol of triethylamine (as a 0·28 M solution in dimethylsulfoxide) and 30 μmol of trichloroacetonitrile (as a 0·6 M solution in dimethylsulfoxide) are added to the sample; the reaction vessel is tightly capped and incubated at 37°C for 1 h. If the reaction mixture fails to turn a deep yellow-orange color (due to excess acid), more triethylamine should be added and the incubation continued. The reaction is terminated by the addition of 1·5 ml of water and the mixture is applied to a column (1 ml) of QAE-Sephadex A50 (formate form), the column is washed with 2 ml of water and eluted with 3 ml of 0·1 N-formic acid. The yield of $^{32}P$-labeled nucleotide in the formic acid eluate, as judged by the amount of $^{32}P$ adsorbed to acid-washed Norite A (Pfanstiehl Inc.) (suspended in 1 N-HCl containing 1% (w/v) $NaH_2PO_4$ and 1% (w/v) $Na_4P_2O_7$), is usually between 60 and 70%. The dimethyl sulfoxide, triethylamine and acetonitrile used in this reaction are redistilled before use and the latter two reagents are stored over $CaH_2$.

The deoxyribonucleoside-5′-monophosphates are converted to the corresponding deoxyribonucleoside-5′-triphosphates with a nucleotide kinase preparation (Symons, 1969). The deoxynucleosides are dried and resuspended in water 4 times and then they are redissolved in 0·2 ml of the kinase reaction mixture (100 mM-Tris·HCl (pH 8·1), 20 mM-$MgCl_2$, 15 mM-KCl, 5 mM-ATP, 0·1 mg bovine serum albumin/ml). To this mixture is added 50 μl phosphoenolpyruvate (100 mM), 10 μl pyruvate kinase (Boehringer Mannheim; 10 mg/ml, 200 units/mg) and 50 μl nucleotide kinase prepared as described by Symons (1969) (the optimum amount will vary from preparation to preparation; in our case 50 μl represented at least a 20-fold excess). The pH of this final reaction mixture is checked and if necessary adjusted to 8·1. The mixture is incubated at 37°C for 1 h and then assayed for deoxyribonucleoside-5′-triphosphates. 2 μl of a 1000-fold dilution of the reaction mixture are mixed with 2 μl of a solution containing 10 mM each of the 5′-mono-, -di- and -triphosphates

of deoxyadenosine and deoxycytosine and half is chromatographed on polyethyleneimine-impregnated cellulose sheets (Polygram CEL 300PEI, Brinkman Instruments Inc.). The chromatogram is developed by ascending chromatography in freshly prepared 0·5 M-$NH_4HCO_3$, the markers are visualized under ultraviolet light and the sections of the chromatogram corresponding to the markers are cut out and counted directly. Generally, more than 45% of the radioactivity is found as dATP and dCTP; the maximum possible value is 65%, as 35% of the monophosphates are 3′, which are not substrates for the nucleotide kinase (Symons, 1969). If insufficient conversion has occurred, additional phosphoenolpyruvate, pyruvate kinase and nucleotide kinase are added, the pH adjusted to 8·1 and the incubation continued.

The deoxynucleoside triphosphate preparation is diluted with 2 ml of water and fractionated on a column (1 ml) of DEAE-Sephadex A50 (bicarbonate form) by successive washes with 2-ml portions of 0·05 M, 0·15 M and 0·75 M-$NH_4HCO_3$. About 90% of the radioactivity in the 0·75 M wash is deoxyribonucleoside-5′-triphosphate. This fraction is diluted with an equal volume of 1 M-triethylamine in 50% (v/v) ethanol and dried by rotary evaporation; it is resuspended in 1 M-triethylamine in 50% ethanol, dried twice more, resuspended in 1 M-$NH_4OH$, dried again and then resuspended in 50% ethanol. The final yield relative to the starting orthophosphate is generally between 15 and 25%.

This method has been used routinely to synthesize a mixture of [$\alpha$-$^{32}$P]dATP and [$\alpha$-$^{32}$P]dCTP. Although the procedure works equally well for [$\alpha$-$^{32}$P]dGTP attempts to synthesize a mixture of labeled dATP, dCTP and dGTP led to over-representation of [$\alpha$-$^{32}$P]dGTP in the product. [$\alpha$-$^{32}$P]dTTP, alone or in combination with other nucleotides, can also be synthesized by this method, but its separation requires paper chromatographic steps which are less convenient and involve considerably greater exposure to high levels of radioactivity.

(d) *Gel electrophoresis*

The electrophoresis buffer contained 89 mM-Tris, 89 mM-$H_3BO_3$ and 2·5 mM-EDTA (pH 8·2) (Greene *et al.*, 1974). Polyacrylamide gels contained 4% (w/v) acrylamide and 0·2% (v/v) *N,N′*-methylenebisacrylamide; electrophoresis was at 100 V for 21 h. The particular conditions used for agarose gel electrophoresis are given in the appropriate Figure legend. DNA bands were stained and visualized as previously described (Shenk *et al.*, 1976).

(e) *$S_1$ nuclease digestion*

Samples (0·5 ml) are removed from reassociation reaction mixtures (containing 1·5 M-NaCl) and diluted into 2·5 ml of 30 mM-sodium acetate (pH 4·55), 0·5 mM-$ZnCl_2$. Samples are digested with excess $S_1$ nuclease at 37°C for 2 h. The $S_1$ nuclease sensitivity of heat-denatured and native nick-translated DNA preparations are assayed similarly.

(f) *Preparation of samples for radioactive counting*

To determine acid-precipitable radioactivity samples were added to 0·1 ml sonicated salmon sperm DNA (500 $\mu$g/ml); 1 ml 1N-HCl containing 1% (w/v) $NaH_2PO_4$ and 1% (w/v) $Na_4P_2O_7$ was then added and the samples incubated for 15 min at 0°C. Precipitates were collected on Whatman GF-C filters, which were washed twice with 2 ml of the acid solution and twice with 2 ml 95% (v/v) ethanol. The filters were dried under a heat lamp and counted using a toluene-based scintillation fluid in a Beckman LS-230 scintillation spectrometer. To determine total radioactivity portions were pipetted directly onto Whatman 3MM filter paper discs which were dried and counted as described above.

## 3. Results

### (a) *Labeling of SV40 DNA with $^{32}$P by nick translation with* Escherichia coli *DNA polymerase I*

Deoxynucleotide residues can be incorporated into DNA by *E. coli* DNA polymerase I provided that a DNA chain with a 3′-hydroxyl terminus is available as primer and there is a suitable template strand to direct the order of deoxynucleotide

addition (Kornberg, 1969). DNA molecules that lack a free 3′-hydroxyl terminus cannot serve as a primer-template for DNA synthesis by this enzyme. However, if nicks with 3′-hydroxyl and 5′-phosphoryl termini are introduced (for example with DNAase I (Young & Sinsheimer, 1965)), DNA polymerase I catalyzes a nick translation reaction, a sequential addition of deoxynucleotide residues to the 3′-hydroxyl end by the polymerase activity and concomitant removal of 5′-phosphoryl residues from the 5′-terminus by the 5′ to 3′ exonuclease activity (Kelly *et al.*, 1970). With excess enzyme the rate of nucleotide replacement is dependent upon the number of nicks introduced by DNAase. The protocol described below permits replacement of 25 to 50% of the unlabeled nucleotides in DNA by labeled nucleotides supplied as deoxynucleoside [$^{32}$P]triphosphates. Generally the labeled DNA has a single-strand length of about 400 nucleotides, an optimal size for hybridization studies (Britten & Kohne, 1968).

The nick translation reaction mixture (120 $\mu$l) contains 50 mM-potassium phosphate (pH 7·4), 5 mM-$MgCl_2$, 15 $\mu$M-dTTP and dGTP and 15 to 20 $\mu$M [$\alpha$-$^{32}$P]dATP and [$\alpha$-$^{32}$P]dCTP (spec. act. 100 to 250 Ci/mmol); generally the DNA concentration in the reaction is about 15 $\mu$g/ml. Activated DNAase I (1·33 ng in 10 $\mu$l; see Materials and Methods) is added for one minute at room temperature, then the mixture is incubated at 14°C with 6 $\mu$g (4 $\mu$l) of DNA polymerase I (spec. act. 18,000 units/mg (Jovin *et al.*, 1969)). Under these conditions the rate of the reaction is limited by the number of nicks. Incorporation of labeled nucleotides into DNA is followed by acid-precipitating and counting suitable portions of the reaction mixture. Following a brief lag, the rate of nucleotide incorporation is nearly linear for one to four hours, then the rate drops to zero and there may even be a loss of labeled nucleotide from the product (Fig. 1), presumably due to the exonuclease activities of DNA polymerase I (Kornberg, 1969). Generally, when net incorporation ceases 60 $\mu$l of 0·25 M-EDTA (pH 7·4) is added and the mixture is heated at 68°C for ten minutes in order to completely inactivate DNAase and DNA polymerase. Figure 1 shows a typical nick

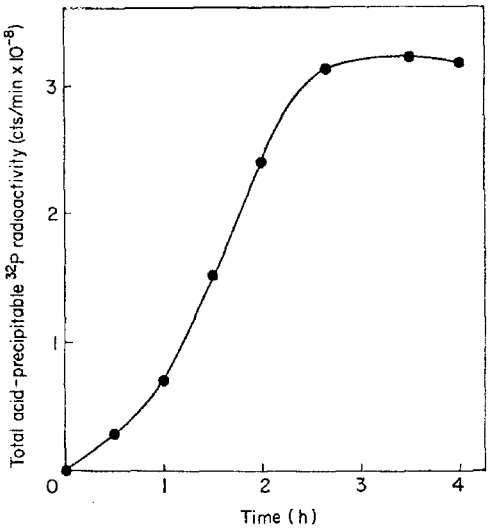


FIG. 1. Time course of nick translation reaction.

The reaction mixture was prepared as described in the text and in this case contained 15 $\mu$M-[$^{32}$P]dATP (96 Ci/mmol), 18 $\mu$M-[$^{32}$P]dCTP (100 Ci/mmol) and 17 $\mu$g SV40 DNA/ml. The reaction was terminated after 4·4 h and the specific activity of the final product was $1{\cdot}45 \times 10^8$ cts/min per $\mu$g.

translation reaction with SV40 DNA in which 37% of the input $^{32}$P-labeled nucleotides were incorporated, corresponding to a replacement (replication) of 44% of the template DNA and a final specific activity of $1{\cdot}45\times10^8$ cts/min per $\mu$g. With deoxynucleoside [$^{32}$P]-triphosphate substrates of specific activity 80 to 130 Ci/mmol the specific activity of the labeled DNA varied from $5\times10^7$ to $2\times10^8$ cts/min per $\mu$g. With substrates of 200 to 250 Ci/mmol DNA specific activities of $10^8$ to $3\times10^8$ cts/min per $\mu$g were achieved.

After removal of a portion for measurement of the specific radioactivity of the labeled DNA, the unreacted triphosphates are removed by passing the mixture over a column (7 ml bed volume) of Sephadex G50 (fine) equilibrated with 10 mM-Tris·HCl (pH 8), 10 mM-NaCl, 2 mM-EDTA. The fractions containing the DNA, detected by counting 1-$\mu$l portions of each, are pooled and stored at 4°C. The DNA concentration in the final preparation is determined by its radioactivity, using the specific activity measured at the end of the reaction. In the vast majority of our experiments concentrations estimated in this way were within 10% of those determined by comparing the reassociation kinetics ($C_0t_{1/2}$)† of the labeled DNA with those of a known concentration of that DNA.

To label larger or smaller amounts of DNA the volume of the reaction mixture is varied proportionately. We have, however, successfully labeled DNA at concentrations as low as 2·5 $\mu$g/ml but in such cases the reaction proceeds more slowly.

### (b) *Characterization of the labeled DNA*

#### (i) *Single-strand length*

The quantity of DNAase I used in the nick translation reaction was chosen to promote substantial rates of nucleotide incorporation and to give a product with an average single-strand length of 400 nucleotides. Because of variations in the activity of commercial DNAase I, and of our stored enzyme preparations, there is some variation in the extent of nicking and, therefore, in the size of the single-strand fragments. Accordingly, each preparation of labeled DNA is sized by sedimentation in alkaline sucrose gradients with suitable marker DNA; an example of such a gradient is shown in Figure 2. If the labeled DNA fragments are larger (500 to 1000 nucleotides), the preparations are sonicated using an MSE Ultrasonicator equipped with a fine probe for two minutes at maximum output, a treatment which reduces the fragments to the desired length. Preparations with mean single-strand fragment lengths less than 300 nucleotides are rare.

#### (ii) *Denaturability of the nick-translated DNA*

Labeled, nick-translated DNA is usually about 90% resistant to digestion by the single-strand-specific $S_1$ nuclease under the conditions described in Materials and Methods, i.e. in 0·28 M-$Na^+$. We attribute the small amount of degradation to the previously recognized "nibbling" of the ends of duplex DNAs (Shenk *et al.*, 1975) and to the occurrence of short single-stranded segments in the nick-translated product.

DNA synthesized by nick translation with DNA polymerase I is generally denaturable, especially when the reaction is carried out at low temperature (Richardson *et al.*, 1964). After denaturation of the labeled DNA (100°C for 3 min followed by

† Abbreviation used: $C_0t_{1/2}$, the product of the total concentration of DNA in a reassociation reaction and the time taken for 50% of that DNA to reassociate.

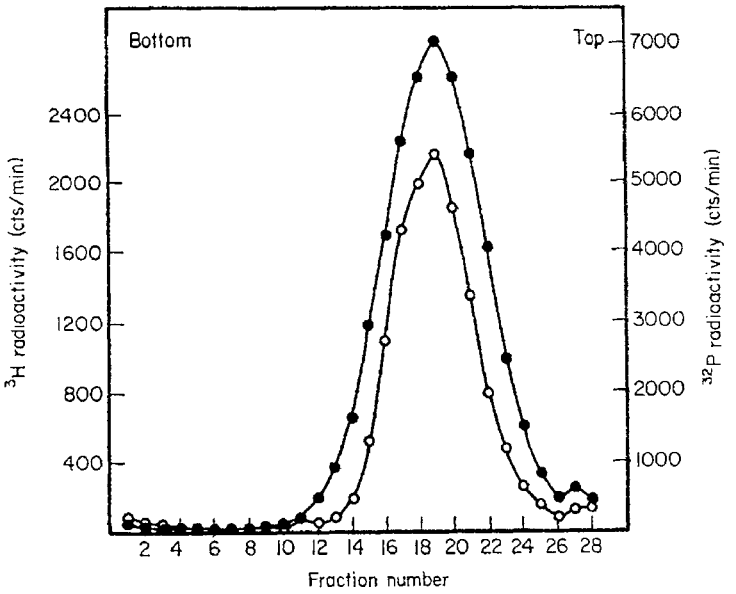


FIG. 2. Determination of single-strand length of DNA labeled by nick translation.

SV40 DNA was labeled by nick translation (—●—●—) to a specific activity of $2{\cdot}45 \times 10^8$ cts/min per $\mu$g. A total of 0·2 ng of this DNA was mixed with 0·4 $\mu$g of sonicated, tritiated polyoma DNA ($5 \times 10^4$ cts/min per $\mu$g) (—○—○—). The mixture of DNAs was sedimented through a 5% to 20% alkaline sucrose gradient in a Beckman SW56 rotor, 55,000 revs/min at 20°C for 6 h. The gradient was dripped onto Whatman 3MM filter paper discs which were counted as described in Materials and Methods. The polyoma DNA marker had a sedimentation coefficient, determined by analytical ultracentrifugation, of 5·7 S, corresponding to a single-strand length of 400 nucleotides (Studier, 1965).

rapid chilling in ice-water) only 1 to 5% is not degraded to acid-soluble products by $S_1$ nuclease. Occasionally, with short linear DNA (500 to 1000 bases), but rarely with circular SV40 DNA as template, as much as 20 to 25% of the denatured nick-translated product is $S_1$ nuclease-resistant. The "undenaturable" DNA is almost certainly molecules with intrastrand complementarity that cause the strands to "snap-back" upon cooling, because after the $S_1$ nuclease digestion, a second cycle of denaturation and cooling converts all the DNA to an $S_1$ nuclease-sensitive form.

Although additional studies are needed to fully explain the origin and variability in occurrence of the rapidly reannealing DNA, our experience suggests that it is most likely related to the differential loss of the 5′ to 3′ exonuclease activity of DNA polymerase I during storage at —20°C. A reduction of 5′ to 3′ exonuclease activity promotes strand displacement and strand switching during synthesis (Schildkraut *et al.*, 1964); the much greater frequency of such anomalous replication with short, linear fragments could depend on the relative frequency with which DNA synthesis is initiated at the fragment's 3′-hydroxyl termini and at nicks.

(iii) *Nick translation yields uniformly labeled DNA*

For nick-translated DNA to be useful in kinetic hybridization experiments the DNA must be labeled throughout at a uniform specific activity. That will be achieved if DNAase I nicks DNA molecules randomly and DNA polymerase I translates each nick with equal probability. To test whether these requirements are met, the DNA was isolated by extraction with phenol after the reaction was terminated with EDTA (see the legend to Fig. 3), cleaved with restriction endonucleases and electrophoresed

on polyacrylamide or agarose gels to obtain fragments corresponding to various parts of the DNA molecule.

With closed circular SV40 DNA as substrate considerable amounts of open circular and linear SV40 DNA remain at the end of the reaction although from the radioactivity throughout the gel an appreciable quantity of smaller, polydisperse DNA fragments are also produced. The linear DNA may well be produced during the nicking step as we have observed that even in the presence of magnesium mild digestion of closed circular DNA with DNAase produces appreciable amounts of linear DNA. The ratio of open circular to linear SV40 DNA in the reaction product is quite variable; in other experiments we have observed a preponderance of the open circular form. Nevertheless, as Figure 3 demonstrates, sequential cleavage of the nick-translated DNA with *Hpa*I and *Eco*RI endonucleases yields the four fragments expected from the cleavage of SV40 DNA by these enzymes.

The quantity of $^{32}P$ label in the largest fragment is less than predicted relative to the smaller fragments. This probably occurs because the linear SV40 DNA arises from scissions at random locations, i.e. it has a circularly permuted sequence and, therefore, there is a greater likelihood of the loss of a large fragment than a small one. For a more quantitative analysis, open circular SV40 DNA was separated from linear DNA and small fragments by sucrose gradient centrifugation, cleaved with either *Hin*dIII, *Hin*cII, *Hin*fI, *Hae*III or *Hpa*I/*Eco*RI restriction endonucleases and the fragments so produced separated by polyacrylamide gel electrophoresis. Bands corresponding to fragments derived from each region of the SV40 genome were cut out and counted; Figure 4(a) shows the results plotted as cts/min *versus* length ($\times 10^{-3}$ bases). The data support the view that nick translation introduces $^{32}P$ label into all parts of the SV40 DNA with equal probability and, therefore, provides a uniformly labeled DNA probe suitable for kinetic hybridization studies.

A similar experiment was carried out with linear $\lambda$ DNA as the substrate for nick translation. After the reaction, full-length $\lambda$ DNA was isolated by sucrose gradient centrifugation, cleaved with *Hin*dIII endonuclease, electrophoresed on an agarose gel to separate six of the seven fragments (the smallest, fragment G, was run off the gel) and the radioactivity in each fragment was determined. Figure 4(b) shows that for all but fragment D the values fall within 20% of the mean specific activity of the DNA. The specific activity of fragment D, the right end of the molecule, is probably higher than the other fragments because, in addition to nucleotide incorporation within the fragment, substantial nucleotide addition at the pre-existing 3′-hydroxyl terminus also occurs. Perhaps fragment A, the left end, does not show this deviation because the probability of nucleotide incorporation within its length is considerably (5 times) greater than for fragment D.

### (c) *Nick-translated DNA is a useful probe for detecting and quantitating rare DNA sequences*

#### (i) *Detection of specific sequences in a complex mixture of restriction fragments*

The detection of rare DNA sequences is frequently limited by the relatively low specific radioactivity of the hybridization probes available. Using the technique of Southern (1975) to transfer DNA from an agarose gel to a nitrocellulose sheet, it is possible by "*in situ*" hybridization to locate specific DNA sequences. By adapting Denhardt's (1966) procedure for DNA/DNA hybridization on nitrocellulose filters to imprints of agarose electrophoretograms on nitrocellulose sheets, sequences present at

the level of one SV40 DNA equivalent per haploid mouse genome can be readily detected with a nick-translated probe. To illustrate this 12·5 $\mu$g of animal cell DNA was mixed with 12·5, 125 or 625 pg of SV40 DNA. These ratios are equivalent to a concentration of 1, 10 and 50 SV40 genome equivalents per haploid mouse genome. The mixed DNAs were digested with *Bam*HI endonuclease, the digests electrophoresed on agarose gels and transferred to nitrocellulose sheets according to Southern (1975). The nitrocellulose sheets, after treatment according to Denhardt (1966), were

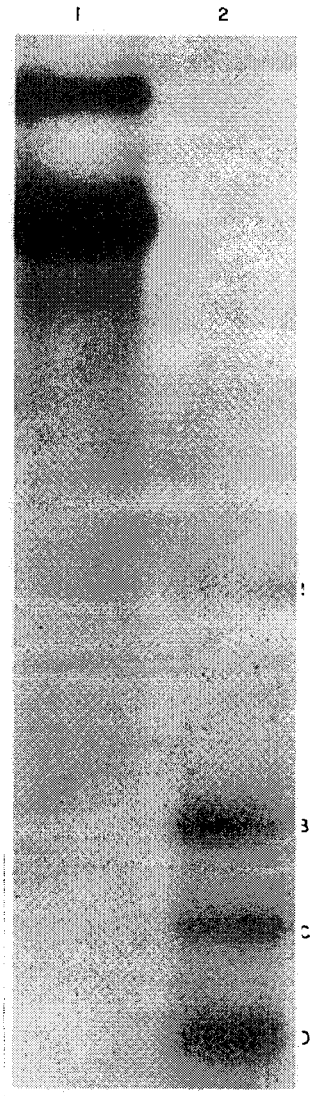


FIG. 3. Gel electrophoresis of undigested and restriction endonuclease-digested, nick-translated SV40 DNA.

Electrophoresis was in a 1·2% (w/v) agarose gel run in a horizontal slab apparatus at 1·2 V/cm for 15 h. The wet gel was radioautographed on Dupont Cronex 4 X-ray film for 6 h and then stained (see Materials and Methods) to permit identification of the bands appearing on the radioautogram.

Track 1: 2 ng of nick-translated SV40 DNA ($5{\cdot}8 \times 10^7$ cts/min per $\mu$g) plus 300 ng unlabeled SV40 DNA. Band A is open-circular SV40 DNA, band B is linear SV40 DNA. Track 2: 1·62 ng of the same nick-translated SV40 DNA was mixed with 400 ng of unlabeled SV40 DNA and digested sequentially with *Hpa*I and *Eco*RI endonucleases. The 4 bands are those expected for this digestion (Sharp *et al.*, 1973). Band A is 38·5% of SV40 (map co-ordinates 0·375 to 0·76), B is 24% (0·76 to 1·0), C is 20% (0·175 to 0·375) and D is 17·5% (0 to 0·175).

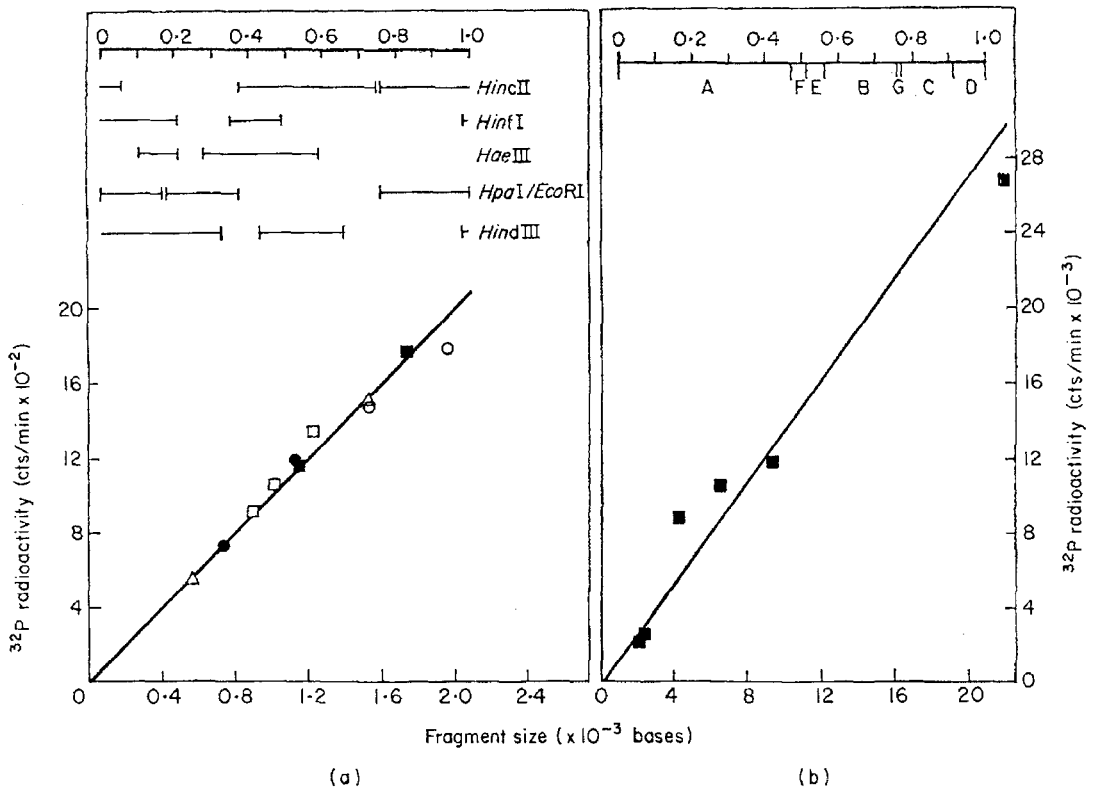


FIG. 4. Nick translation labels DNA uniformly.

(a) Closed circular SV40 DNA was labeled by nick translation as described in the text except that the reaction was terminated by the addition of EDTA followed by extraction with phenol. The product had a spec. act. of $5{\cdot}3 \times 10^7$ cts/min per $\mu$g. A total of 1·4 $\mu$g of this material was mixed with 4$\mu$g unlabeled SV40 DNA and open circular SV40 DNA was isolated by sedimentation through a 5% to 20% neutral sucrose gradient containing 1 M-NaCl (Beckman SW41 rotor, 40,000 revs/min at 4°C for 8 h). This DNA was precipitated with ethanol, resuspended in 10 mM-Tris·HCl (pH 8·0), 10 mM-NaCl, 2 mM-EDTA and dialyzed against this buffer. Electrophoresis in a 1·2% (w/v) agarose gel showed that more than 90% of the radioactivity migrated at the position of open circular SV40 DNA. Then 12 ng samples of $^{32}$P-labeled, nick-translated open circular SV40 DNA were mixed with 160 ng SV40 DNA and digested with the restriction endonucleases *Hinc*II (○), *Hind*III (■), *Hinf*I (●), *Hae*III (△) and sequentially with *Hpa*I and *Eco*RI (□). The resulting fragments were fractionated by polyacrylamide gel electrophoresis. The DNA bands were visualized as described in Materials and Methods, cut out of the gel and counted as described by Carbon *et al.* (1975).

The sizes and locations of the restriction endonuclease fragments are taken from the following references:

| | |
|---|---|
| *Hinc*II, *Hind*III | Danna *et al.* (1973); Lai & Nathans (1974). |
| *Hinf*I | K. N. Subramanian, S. M. Weissman, B. S. Zain and R. J. Roberts (personal communication). |
| *Hae*III | Yang *et al.* (1976). |
| *Hpa*I | Sharp *et al.* (1973). |

(b) Phage $\lambda$ DNA was labeled by nick translation as described in the text except that one-tenth the normal amount of DNAase was used and the reaction was terminated by the addition of EDTA followed by extraction with phenol. The product has a spec. act. of $1{\cdot}4 \times 10^7$ cts/min per $\mu$g. Then 0·6 $\mu$g of this material was mixed with 2·1 $\mu$g unlabeled $\lambda$ DNA and full-length $\lambda$ DNA was purified by sedimentation through a 5% to 20% neutral sucrose gradient containing 1 M-NaCl (Beckman SW41 rotor, 40,000 revs/min at 4°C for 6 h). This DNA was precipitated with ethanol, resuspended in 10 mM-Tris·HCl (pH 8·0), 10 mM-NaCl, 2 mM-EDTA and dialyzed against this buffer. Electrophoresis of this material in a 0·7% (w/v) agarose gel showed that all of the radioactivity migrated at the position of full-length $\lambda$ DNA. Approximately 0·3 $\mu$g of this material was digested with *Hind*III endonuclease and the digest was electrophoresed on a 0·7% (w/v) agarose cylindrical gel (0·4 cm × 23 cm) at 55 V for 18 h. The DNA bands were visualized as described in Materials and Methods, cut out of the gel and counted by Cerenkov radiation. The sizes and locations of the restriction endonuclease fragments are taken from Murray & Murray (1975).

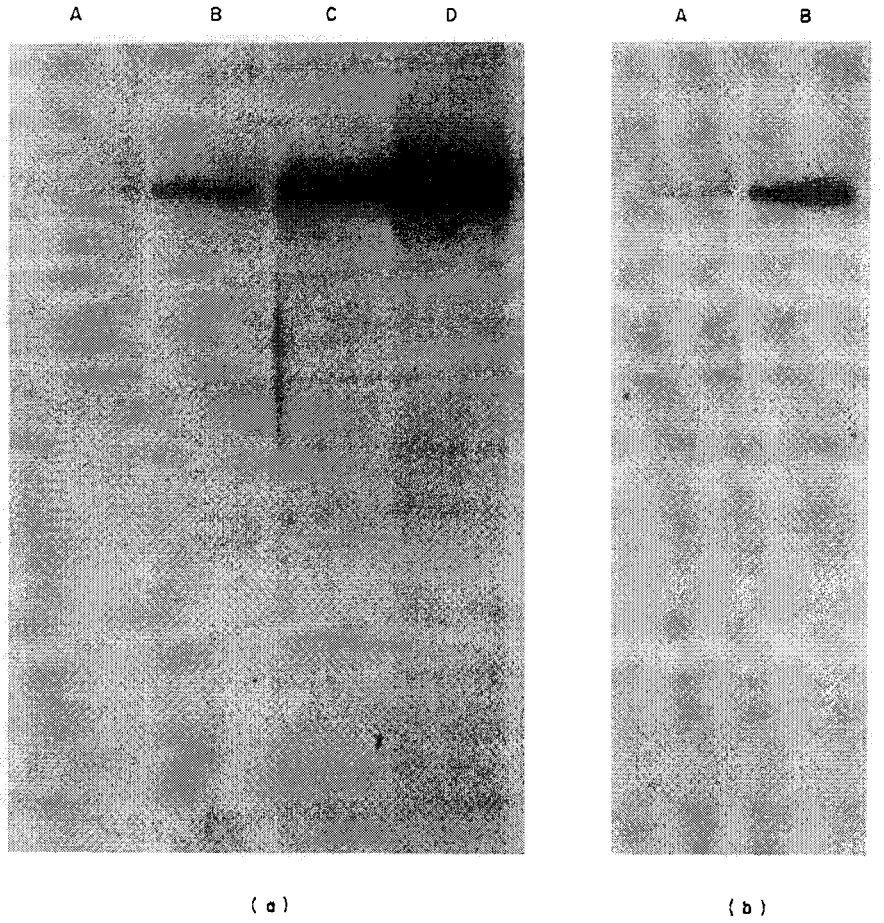


FIG. 5. Radioautographic detection of sequences present at the level of one copy per haploid mouse genome.

A total of 12·5 $\mu$g of *D. melanogaster* DNA was mixed with 12·5, 125 or 625 pg of SV40 DNA and the mixtures digested with *Bam*HI endonuclease. These mixtures simulate concentrations of 1, 10 and 50 SV40 DNA equivalents per haploid mouse genome. The digests were electrophoresed in a 0·7% (w/v) agarose gel run in a horizontal slab apparatus at 0·8 V/cm for 16·5 h. The DNA was then transferred to nitrocellulose strips (Southern, 1975) which were then baked under vacuum at 80°C for 2 h and incubated in PM (0·02% Ficoll, 0·02% polyvinylpyrollidone, 0·02% bovine serum albumin) in 6 × SSC (SSC is 0·15 M-NaCl, 0·015 M-Na citrate, pH 7·0) at 65°C for 8·5 h (Denhardt, 1966). The treated strips were then hybridized with $^{32}$P-labeled, nick-translated SV40 DNA ($2{\cdot}8 \times 10^7$ cts/min per $\mu$g). The hybridization solution (4 ml) contained 0·14 $\mu$g SV40 DNA and 800 $\mu$g sonicated salmon sperm DNA in 6 × SSC plus PM. Hybridization was at 65°C for 34 h. The strips were then rinsed in 2 × SSC and washed at 65°C in the hybridization buffer for 1 h; in 2 × SSC, 0·1 M-potassium phosphate (pH 7) for 1 h; and in 2 × SSC for 2 h. They were then blotted dry and radioautographed.

Track A: 1 SV40 equivalent per haploid mouse genome (12·5 pg SV40 DNA). Track B: 10 SV40 equivalents (125 pg). Track C: 50 SV40 equivalents (625 pg). Track D: linear SV40 DNA marker (2 ng). (a) Radioautographed on Dupont Cronex 4 X-ray film for 5 days; (b) showing only tracks A and B, was radioautographed on Kodak XR-5 X-ray film using a Kodak regular intensifying screen (R. A. Laskey & A. D. Mills, personal communication) for 3 days.

incubated with $^{32}$P-labeled nick-translated SV40 DNA (spec. act. $2{\cdot}8 \times 10^7$ cts/min per $\mu$g), washed free of adventitiously bound label and radioautographed. Figure 5 shows that the detection of a DNA sequence present at one part per $10^6$ is achievable with a 12·5 $\mu$g DNA sample using a moderately labeled, nick-translated DNA probe. With more highly labeled probes ($2 \times 10^8$ cts/min per $\mu$g) and longer radioautographic exposures, even greater sensitivity of detection can be achieved.

(ii) *Quantitation of specific sequences by reassociation kinetics*

Measurement of DNA/DNA reassociation kinetics provides a convenient, straightforward and sensitive way to estimate the concentration of homologous DNA sequences in the presence of a great excess of unrelated DNA (Gelb *et al.*, 1971). The availability of high specific activity $^{32}$P-labeled SV40 DNA has enabled us to simplify the determination of the amount of SV40 DNA in SV40-transformed mouse cells (Gelb *et al.*, 1971; Botchan *et al.*, 1974). Here we illustrate that reassociation kinetic measurements with DNA probes of specific activity about $7 \times 10^7$ cts/min per $\mu$g can readily detect one SV40 DNA equivalent per haploid mouse genome using only 100 $\mu$g of cellular DNA.

Denatured $^{32}$P-labeled nick-translated SV40 DNA (35 pg/ml) was reassociated in the presence and absence of transformed cell DNA (31 $\mu$g/ml). Reassociation was monitored with $S_1$ nuclease (Sutton, 1971) which degrades unreacted single-stranded DNA and leaves the reassociated sequences acid-precipitable. Because the labeled SV40 DNA is randomly sheared, the reassociation reaction follows pseudo second-order kinetics (Morrow, 1974); by using appropriate corrections of the data (Morrow, 1974; Morrow *et al.*, manuscript in preparation) linear plots can be obtained. Figure 6 shows that the addition of unlabeled SV40 DNA (78 pg/ml) increased the reassociation rate threefold as expected; the addition of 31 $\mu$g of transformed cell DNA per ml increased the reassociation rate twofold indicating the presence of 1·6 SV40 genome equivalents per haploid mouse genome (assuming that the mouse genome is $1{\cdot}33 \times 10^6$ times the size of the SV40 genome (Gelb *et al.*, 1971)). With nick-translated SV40 DNA of the highest specific activity we have prepared ($3 \times 10^8$ cts/min per $\mu$g) the quantity of cellular DNA needed to achieve this level of sensitivity is reduced proportionately (data not shown).

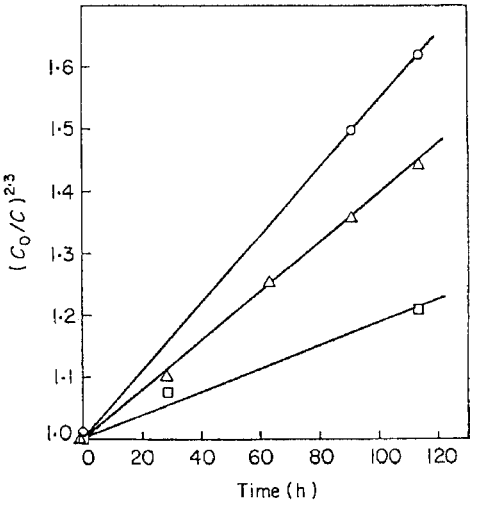


FIG. 6. DNA/DNA reassociation kinetics using SV40 DNA labeled by nick translation. The reaction mixtures contained 10 mM-Tris·HCl (pH 7·5), 2 mM-EDTA, 1·5 M-NaCl and 250 $\mu$g sonicated salmon sperm DNA/ml, together with the indicated amounts of SV40 or transformed cell DNA in a total volume of 3·25 ml. Reassociation, at 68°C, was monitored with $S_1$ nuclease (Sutton, 1971); 0·5-ml samples were diluted into 2·5 ml $S_1$ nuclease digestion buffer, stored at −20°C and processed as described in Materials and Methods. The reaction mixtures contained: SV40 [$^{32}$P]DNA ($6{\cdot}7 \times 10^7$ cts/min $\mu$g), 35 pg/ml (—□—□—); plus SV40 DNA 78 pg/ml (—○—○—); plus SV3T3 C126 (C. N. Cole, P. W. J. Rigby & P. Berg, unpublished observations) DNA, 31 $\mu$g/ml (—△—△—). All nucleic acids had a single-strand length of 400 nucleotides.

In assays of this type only the initial rate of reassociation is measured as this is sufficient for accurate determination of the concentration of SV40 sequences in the cellular DNA sample (Morrow *et al.*, manuscript in preparation). However, extensive experience with the use of nick-translated DNA probes in reassociation kinetics experiments shows that all of the labeled DNA can be driven into hybrid form by excess unlabeled DNA. The kinetics of reassociation of nick-translated DNA are identical to those obtained with DNA labeled *in vivo*, indicating that both strands are equally represented (Morrow, 1974).

## 4. Discussion

*E. coli* DNA polymerase I's ability to catalyze a nick translation reaction (Kelly *et al.*, 1970) has been used to label circular and linear DNAs to high specific radioactivities ($>10^8$ cts/min per $\mu$g DNA). Although the nucleotide replacement does not exceed 40 to 50%, the DNA product is uniformly labeled throughout, indicating that the introduction of nicks and initiation of nick translation occurs with equal probaability within all regions of the DNA. While there is evidence that DNAase I has sequence specificity (Ehrlich *et al.*, 1973) the sequences recognized are sufficiently small that they will be distributed randomly with respect to the overall geography of the DNA molecule. Moreover, any bias in the nicking reaction will be obscured because polymerase I can translate nicks much further than the average distance between nicks. Upon denaturation the labeled DNA yields single-strand fragments approximately 400 nucleotides long, an optimal length for use in hybridization experiments (Britten & Kohne, 1968).

An important element in the *in vitro* labeling procedure is the availability of high specific activity deoxynucleoside triphosphates. These are readily prepared by a modification of Symons' (1969) procedure which is simple because it uses inorganic phosphoric acid and unprotected nucleosides in the synthesis. By adjusting the ratio of $^{32}$P-labeled phosphate to unlabeled carrier phosphate the specific activity of the deoxynucleoside triphosphates, and therefore the DNA, can be modified to suit the needs of the experiment; in this way Jaenisch & Mintz (1974) prepared labeled substrates at 1000 Ci/mmol and labeled SV40 DNA to $6\times10^8$ cts/min per $\mu$g in order to search for SV40 DNA sequences in the DNA from a variety of animal tissues. DNA with even higher specific activity could be prepared using $^{125}$I-labeled deoxycytosine triphosphate (Scherberg & Refetoff, 1974) as a substrate for the DNA polymerase I-catalyzed nick translation reaction. This procedure also avoids some of the problems, e.g. cross-linking, associated with direct iodination. Where it has been desirable to prepare labeled DNA that does not decay quickly, we have used commercially available [$^3$H]deoxynucleoside triphosphates (spec. act., 20 to 60 Ci/mmol); with such substrates SV40 DNA has been labeled to $2 \times 10^7$ cts/min per $\mu$g, a level about ten times higher than is usually obtained by labeling of SV40 DNA *in vivo* with $^{32}$P.

Besides SV40 and $\lambda$ phage DNAs, polyoma, adenovirus 2, $\phi$X174 and M13 DNAs have been labeled in our laboratory by the same nick-translation reaction protocol. DNAs from adenovirus 12 (Green *et al.*, 1976*a*,*b*), herpes simplex virus (Frenkel *et al.*, 1976) and the human papova virus BK (G. di Mayorca, personal communication) have been labeled to high specific activity by the same procedure in other laboratories. Hogness and his colleagues have used nick translation to label "Thomas circles"

(Schachat & Hogness, 1973), hybrid bacterial plasmid–*Drosophila* DNAs and total *Drosophila* DNA in their studies of DNA sequence organization in *D. melanogaster* chromosomes (Wensink *et al.*, 1974; Glover *et al.*, 1975). A particularly interesting application is the successful labeling of human hepatitis B virus DNA by nick translation (Rigby, Landers, Greenberg & Robinson, unpublished observations) because the absence of a suitable cell culture system for growing this virus precludes its labeling *in vivo*.

The availability of highly labeled DNA extends the sensitivity of hybridization reactions for detecting rare nucleic acid sequences. With nick-translated SV40 DNA containing $10^8$ cts/min per $\mu$g, one SV40 DNA equivalent per cell genome can be detected readily. Screening large numbers of clones of SV40 transformed cells for the number of integrated SV40 copies is thereby made easier and more economical. With DNAs labeled to such high specific activities restriction fragments containing unique, single copy sequences can be detected by *in situ* hybridization after their transfer to nitrocellulose strips according to Southern (1975). Hogness' laboratory (Wensink *et al.*, 1974; Glover *et al.*, 1975) has already demonstrated how *in situ* hybridization of labeled nick-translated DNA to *Drosophila* chromosomal squashes enabled them to identify the polytene chromosomal location of cloned segments of *Drosophila* DNA.

This research was supported in part by grants from the United States Public Health Service (GM-13235-10) and the American Cancer Society (VC-23D). One of us (P. W. J. R.) was a Fellow of the Helen Hay Whitney Foundation, another author (C. R.) was a Dernham Fellow of the American Cancer Society, California Division.

## REFERENCES

Bernardi, G. (1965). *Nature (London)*, **206**, 779–783.
Bolton, E. T. & McCarthy, B. J. (1962). *Proc. Nat. Acad. Sci., U.S.A.* **48**, 1390–1397.
Botchan, M., Ozanne, B., Sugden, B., Sharp, P. A. & Sambrook, J. (1974). *Proc. Nat. Acad. Sci., U.S.A.* **71**, 4183–4187.
Botchan, M., Topp, W. & Sambrook, J. (1976). *Cell*, **9**, 269–287.
Britten, R. J. & Kohne, D. E. (1968). *Science*, **161**, 529–540.
Carbon, J., Shenk, T. E. & Berg, P. (1975). *Proc. Nat. Acad. Sci., U.S.A.* **72**, 1392–1396.
Danna, K. J., Sack, G. H. & Nathans, D. (1973). *J. Mol. Biol.* **78**, 363–376.
Denhardt, D. J. (1966). *Biochem. Biophys. Res. Commun.* **23**, 641–646.
Ehrlich, S. D., Bertazzoni, V. & Bernardi, G. (1973). *Eur. J. Biochem.* **40**, 143–147.
Frenkel, N., Locker, H., Cox, B., Roizman, B. & Rapp, F. (1976). *J. Virol.* **18**, 885–893.
Gelb, L. D., Kohne, D. E. & Martin, M. A. (1971). *J. Mol. Biol.* **57**, 129–145.
Gillespie, D. & Spiegelman, S. (1965). *J. Mol. Biol.* **12**, 829–842.
Glover, D. M., White, R. L., Finnegan, D. J. & Hogness, D. S. (1975). *Cell*, **5**, 149–157.
Green, M. R., Chinnadurai, G., Mackey, J. K. & Green, M. (1976*a*). *Cell*, **7**, 419–428.
Green, M. R., Green, M. & Mackey, J. K. (1976*b*). *Nature (London)*, **261**, 340–342.
Greene, P. J., Betlach, M. C., Goodman, H. M. & Boyer, H. W. (1974). In *Methods in Molecular Biology* (Wickner, R. B., ed.), vol. 7, pp. 87–111, Marcel Dekker Inc., New York.
Gross-Bellard, M., Oudet, P. & Chambon, P. (1973). *Eur. J. Biochem.* **36**, 32–38.
Hall, B. D. & Spiegelman, S. (1961). *Proc. Nat. Acad. Sci., U.S.A.* **47**, 137–146.
Jaenisch, R. & Mintz, B. (1974). *Proc. Nat. Acad. Sci., U.S.A.* **71**, 1250–1254.
Jovin, T. M., Englund, P. T. & Bertsch, L. L. (1969). *J. Biol. Chem.* **244**, 2996–3008.
Kelly, R. B., Cozzarelli, N. R., Deutscher, M. P., Lehman, I. R. & Kornberg, A. (1970). *J. Biol. Chem.* **245**, 39–45.
Kornberg, A. (1969). *Science*, **163**, 1410–1418.
Lai, C. J. & Nathans, D. (1974). *J. Mol. Biol.* **89**, 179–193.

Maniatis, T., Jeffrey, A. & Kleid, D. G. (1975). *Proc. Nat. Acad. Sci., U.S.A.* **72**, 1184–1188.
Morrow, J. F. (1974). Ph.D. thesis, Stanford University, Stanford.
Murray, K. & Murray, N. E. (1975). *J. Mol. Biol.* **98**, 551–564.
Noyes, B. E. & Stark, G. R. (1975). *Cell*, **5**, 301–310.
Reed, S. I., Stark, G. R. & Alwine, J. C. (1976). *Proc. Nat. Acad. Sci., U.S.A.* **76**, 3083–3087.
Richardson, C. C., Inman, R. B. & Kornberg, A. (1964). *J. Mol. Biol.* **9**, 46–69.
Roberts, R. J., Breitmeyer, J. B., Tabaeknik, N. F. & Myers, P. A. (1975). *J. Mol. Biol.* **91**, 121–123.
Schachat, F. H. & Hogness, D. S. (1973). *Cold Spring Harbor Symp. Quant. Biol.* **38**, 371–381.
Scherberg, N. H. & Refetoff, S. (1974). *Biochim. Biophys. Acta*, **340**, 446–451.
Schildkraut, C. L., Richardson, C. C. & Kornberg, A. (1964). *J. Mol. Biol.* **9**, 25–45.
Sharp, P. A., Sugden, B. & Sambrook, J. (1973). *Biochemistry*, **12**, 3055–3063.
Shenk, T. E., Rhodes, C., Rigby, P. W. J. & Berg, P. (1975). *Proc. Nat. Acad. Sci., U.S.A.* **72**, 989–993.
Shenk, T. E., Carbon, J. & Berg, P. (1976). *J. Virol.* **18**, 664–671.
Smith, H. S., Turner, S., Leong, J. A. & Rigby, P. W. J. (1976). *J. Virol.* **19**, 146–153.
Southern, E. M. (1975). *J. Mol. Biol.* **98**, 503–517.
Studier, F. W. (1965). *J. Mol. Biol.* **11**, 373–390.
Sutton, W. D. (1971). *Biochim. Biophys. Acta*, **240**, 522–531.
Symons, R. H. (1969). *Biochim. Biophys. Acta*, **190**, 548–550.
Thomas, M. & Davis, R. W. (1974). *J. Mol. Biol.* **91**, 315–328.
Vogt, V. M. (1973). *Eur. J. Biochem.* **33**, 192–200.
Wensink, P. C., Finnegan, D. J., Donelson, J. E. & Hogness, D. S. (1974). *Cell*, **3**, 315–325.
Yang, R. C., Van De Voorde, A. & Fiers, W. (1976). *Eur. J. Biochem.* **61**, 101–117.
Young, E. T. & Sinsheimer, R. L. (1965). *J. Biol. Chem.* **240**, 1274–1280.